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Exposure of *Agaricus bisporus* to *Trichoderma aggressivum* f. *europaeum* leads to growth inhibition and induction of an oxidative stress response.

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Key words: *Agaricus bisporus*, *Trichoderma aggressivum*, proteomics, host-pathogen interactions.

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List of abbreviations: FDR: False Discovery Rates; GO: gene ontology; SSDA: statistically significant differentially abundant; DEP: differentially expressed proteins; ME: malt extract;

CYM: complete yeast media; SN: supernatant; MPN: Most probably number; RH: Relative humidity; SE: Standard error

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Abstract

Green mould disease of mushroom, *Agaricus bisporus*, is caused by *Trichoderma* species and can result in substantial crop losses. *Trichoderma aggressivum* supernatant inhibited growth of *A. bisporus* by 28% after 2 days, 48% after 4 days ($P = 0.05$), and by 13% after 8 days, respectively. Label free proteomic analysis of changes in the abundance of *A. bisporus* proteins following exposure to *T. aggressivum* supernatant *in vitro* indicated increased abundance of proteins associated with an oxidative stress response (zinc ion binding (+6.6 fold); peroxidase activity (5.3-fold); carboxylic ester hydrolase (+2.4 fold); dipeptidase (+3.2 fold); [2Fe-2S] cluster assembly (+3.3 fold)). Proteins that decreased in relative abundance were associated with growth: structural constituent of ribosome, translation (-12 fold), deadenylation-dependent decapping of nuclear-transcribed mRNA (-3.4 fold), small GTPase mediated signal transduction (-2.6 fold), deoxyribonucleotide catabolic process (-2.6 fold), GTP binding (-2.7 fold), glycine cleavage system P protein (-2.3 fold), and proteasome subunit beta (-2.3 fold). *In vivo* analysis revealed that 10^{-4} (0.1 g/kg) *T. aggressivum* inoculum decreased the mushroom yield by 29% to 56% and 10^{-3} (1 g/kg) *T. aggressivum* inoculum decreased the mushroom yield by 68% to 100%. Green mould was observed on inoculated plots from day 14 after casing, characterized by dense white mycelium that turned green after extensive sporulation. Proteins that increased in abundance in *A. bisporus in vivo* following exposure to *T. aggressivum* indicated an oxidative stress response and included proteins with pyruvate kinase activity (+2.6 fold) and hydrolase activity (+2.1 fold)). Proteins decreased in relative abundance were involved in polysaccharide catabolic process, formation of extracellular region and lyase activity (-3 fold). The results indicate that exposure of *A. bisporus* mycelium to *T. aggressivum in vitro* and *in vivo* resulted in an oxidative stress response and reduction in growth.

Introduction

The annual global production of edible mushrooms is estimated to be worth approximately \$42 billion dollars (Prescott *et al.* 2018), with the white button mushroom (*Agaricus bisporus*) being the most commonly cultivated edible mushroom in North America and Europe (Royse *et al.* 2017) and the main horticultural crop in the Republic of Ireland (Alexander 2015). The most important fungal pathogens of edible mushrooms worldwide are species belonging to genera *Lecanicillium*, *Mycogone*, *Cladobotryum* and *Trichoderma* (Fletcher & Gaze, 2008; Potocnik *et al.* 2015). Five decades ago, *Lecanicillium fungicola*, *Mycogone perniciosa*, and *Cladobotryum* sp., causal agents of dry and wet bubble, and cobweb disease respectively, were the major mycopathogens damaging mushroom production (Forer *et al.* 1974). *Trichoderma* spp., such as *T. harzianum*, *T. atroviride*, *T. koningii*, *T. virens*, etc., have been present in mushroom industry for many years (Sinden and Hauser 1953; Fletcher and Gaze 2008), but in 1980s a new substrate-colonising aggressive species that severely affected mushroom production was found in Ireland (Seaby 1987, 1996) and was subsequently named *T. aggressivum* (Samuels *et al.* 2002). It first appeared in two sites, in the British Isles (*T. aggressivum* f. *europaeum*) and North America (*T. aggressivum* f. *aggressivum*), and then spread worldwide causing a worldwide green mold epidemic (Rinker 1993; Romaine *et al.* 1996; Geels 1997; Hermosa *et al.* 1999; Mamoun *et al.* 2000; Hatvani *et al.* 2007; Szczech *et al.* 2008; Romero-Arenas *et al.* 2009; Clift and Shamshad 2009; Hatvani *et al.* 2012; Kosanovic *et al.* 2013) and enormous economic losses (Kredics *et al.* 2010). This filamentous fungus is a rapidly growing, aggressive competitor that has evolved from *T. harzianum* and adapted to a specific mushroom substrate growing niche (Kredics *et al.* 2010; Kosanovic *et al.* 2015). Unlike *T.*

88 *harzianum*, *T. aggressivum* colonizes the substrate for *A. bisporus* growth and causes crop losses
 89 of between 60% and 100% (O'Brien *et al.* 2017; Seaby 1996; Kredics *et al.* 2010). Necrotic
 90 brown spots on mushrooms may occur, while during serious outbreaks, the crop completely fails,
 91 since no fruiting bodies develop (Seaby 1996). Disease control on mushrooms farms includes the
 92 application of only a small number of fungicides and, due to the long-term or continuous usage
 93 of fungicides, the development of fungicide-resistance has become a common phenomenon
 94 (Fletcher & Yarham 1976; Grogan & Gaze 2000). While *T. aggressivum* was shown to be
 95 controlled by the benzimidazole fungicide - carbendazim in the mid-1990s (Grogan *et al.* 1996),
 96 resistance of *Trichoderma aggressivum* to the benzimidazole fungicide: benomyl and
 97 thiophanate-methyl was detected in North American mushroom farms in the early 2000s
 98 (Romaine *et al.* 2005).

100 The aim of this study was to characterize the interaction of *T. aggressivum* with *A.*
 101 *bisporus*. Microscopy and enzyme studies suggested competitive saprophytic interaction
 102 (Williams *et al.* 2003), but recent advances in proteomic technologies can produce novel insights
 103 into host-pathogen interactions not visible by more traditional methods. These technologies
 104 include large scale mass spectrometry-based proteomics capable of identifying and quantifying
 105 thousands of proteins across multiple samples in a single run (Zhang *et al.* 2013; Yates *et al.*
 106 2009; Watson *et al.* 2015; Zhu *et al.* 2013; Barribeau *et al.* 2014). O'Brian *et al.* 2014 examined
 107 the proteomic response of *T. aggressivum* to *A. bisporus*, but this current study focuses on how
 108 *Agaricus* responds to the pathogen, since the pathogen *T. aggressivum* and *A. bisporus* are both
 109 present in the substrate at the same time and fungal – fungal interactions may be important

factors in disease development in the crop and offer new insights into the development of novel control strategies.

Materials and Methods

Fungal culture

T. aggressivum f. *europaeum* 100526 (acquired from the Royal Netherlands Academy of Arts and Science, Utrecht, Netherlands) preserved in liquid nitrogen culture collection of Maynooth University. PCR analysis was performed to double check the green mould re-isolated from the substrate and used as an inoculum subsequently. Three sets of primers were used (**18S INT** – 5'TAA CAA CAC GCC TGC TTA AGA'3; **TH1 INT REV** – 5'GAG AAG GCT CAG ATA GTA AAA AAT'3; **TH1 INT** – 5'CCC CCT CGC GGG GTT ATT TTT ACT'3; **EX ITS1** – 5'GTA ACA AGG TTT CCG TAG GTG'3; **EX ITS4** – 5'TTC TTT TCC TCC GCT TAT TGA TTG'3), general (ITS1/4) for *Trichoderma* spp., TH1 INT/ITS4 for *T. aggressivum*, and 18S/TH1 INT REV for subspecies *T. aggressivum* f. *europium* (Fig. S3) The results confirmed that re-isolated sample from virulence test was *T. aggressivum* f. *europaeum*. *Trichoderma* stocks were maintained on malt extract (ME) agar (Oxoid).

A. bisporus commercial strain A15 (Sylvan Inc., France) was used in this study. *A. bisporus* was maintained as spawn (sterilized rye grains colonized with *A. bisporus*) and on CYM agar plates.

Effect of *T. aggressivum* supernatants on growth of *A. bisporus* *in vitro*

Trichoderma cultures (10^4 conidia/ml) were grown in malt extract (ME) broth (Oxoid) at 30 °C and 200 rpm for 2 days, centrifuged and filter-sterilized through 0.2 µm syringe filter to produce supernatants. Cultures (50 ml) of *A. bisporus* (one spawn grain of A15 in CYM broth) were grown for 10 days at 25°C and 200 rpm and supplemented with 25% v/v supernatant of *T. aggressivum* for an additional 2-, 4- or 8-days co-incubation at 25°C and 200 rpm. *A. bisporus* mycelium was harvested by centrifugation at 4143 g for 15 min. The supernatant was discarded, and wet mass was measured.

***T. aggressivum* inoculum preparation for an *in vivo* test**

Mushroom substrate was infected with *T. aggressivum* for an *in vivo* cropping trial according to the method by O'Brien *et al.* (2017). Briefly, inoculum was prepared for a cropping trial by inoculating Phase II mushroom substrate with *T. aggressivum* conidia. Six spawn grains were coated with $\sim 10^6$ *T. aggressivum* conidia per grain at spawning time and added to 18kg Phase II mushroom substrate, along with 90g of fresh spawn (0.5 % rate). At the Teagasc Experimental Mushroom Unit (Ashtown, Co. Dublin, Ireland) mushroom growing crates (45L, 600 x 400 x 235 mm) were filled with substrate, covered and incubated under standard spawn-run conditions (25°C and 95% RH) to produce Phase III substrate which was heavily colonised by *T. aggressivum* (Figure S1).

Samples were taken to calculate the CFU/g of *T. aggressivum* inoculum according to the method described by O'Brien *et al.* (2017). Homogenized substrate was diluted from 10^{-1} to 10^{-9} in sterile water and spotted onto plates. The initial fungal load in the inoculum was estimated in terms of CFUs/g of fresh weight by using the most probable number (MPN) method (Phelps 1908). Inoculum contained 4.5×10^8 *Trichoderma* CFUs/g (Figure S2) and the *Trichoderma*

present was confirmed as *T. aggressivum* f. *europaeum* by PCR (Figure S3) (O'Brien *et al.* 2017).

Effect of *T. aggressivum* infected substrate on growth of *A. bisporus* *in vivo*

T. aggressivum inoculum was added to 16 kg crates of uninfected Bulk Phase III mushroom substrate, fully colonised with *A. bisporus*, at two rates: 1 g/kg and 0.1 g/kg, equivalent to a dilution level of 10^{-3} and 10^{-4} infected substrate by weight. A control treatment of uninfected Phase III substrate was also prepared. Substrate was sampled during the first flush and the yield was harvested up to second flush. The experimental trial at the Teagasc Experimental Mushroom Unit was set up according to a randomised block design consisting of three inoculation treatments: control, 10^{-3} and 10^{-4} and five time points: day 10, 15, 20, 25 and the end of second flush (day 35) which was the end of the trial. Four replicates were prepared for each treatment combination. Compost samples for proteomics analysis were disruptively collected from the first four time point plots on days 10, 15, 20 and 25. Data on the effect of inoculum dilution level on mushroom yield for two flushes was collected from the remaining intact plots (day 35). Disease symptoms: green mould occurrence on casing and compost, deformities and spots on fruiting bodies, and pilei colorimetry assay (Chroma Meter CR-400, Konica Minolta, Sensing, INC., Japan) were also recorded from these plots.

Label free quantitative proteomics of *A. bisporus* treated with *T. aggressivum*

Proteins were extracted according to Maher *et al.*, (2018) from *A. bisporus* mycelium which was grown *in vitro* for 10-days and then supplemented with *T. aggressivum* supernatant (n

= 4) for an additional 4-days. *A. bisporus* proteins were also extracted from compost mycelium (*in vivo* testing) which was grown together with *T. aggressivum* at two different concentrations, 10^{-3} and 10^{-4} inoculum ($n = 4$), for 25-days in a mushroom growing room. The Bradford method was used to quantify proteins for acetone precipitation overnight. Samples were centrifuged at 14500 g for 10 min, and the pellet was re-suspended in 25 μ l of 6 M urea, 2 M thiourea and 0.1 M Tris-HCl buffer (pH 8.0). Proteins were reduced with dithiothreitol (0.5 M DTT), alkylated with iodoacetamide (0.55 M IAA) and digested with sequence grade trypsin (Promega, Ireland) at a trypsin: protein ratio of 1:40, overnight at 37°C. Tryptic peptides were purified for mass spectrometry using C18 spin columns (Medical Supply Company, Ireland) and 0.75 μ g of peptide mix was eluted onto a QExactive (ThermoFisher Scientific, USA) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated by an increasing acetonitrile gradient from 2%–40% on a Biobasic C18 Picofrit column (100 mm length, 75 mm ID), using a 120-min reverse phase gradient at a low rate of 250 nl/min. A full MS scan of range 200–2000 was followed to select the 15 most intense ions prior to MS/MS. Identification of proteins from this data was performed using the Andromeda search engine in Max-Quant (version 1.2.2.5) to correlate against a *A. bisporus* database downloaded from www.uniprot.org.

The MS proteomic data and MaxQuant search output files have been added to the ProteomeXchange Consortium (Côté *et al.*, 2012) via the PRIDE partner repository with the dataset identifier PXD016719. The Perseus software package (v. 1.5.5.3) was used for results processing, statistical analyses and graphics generation. LFQ intensities were log2-transformed and ANOVA of significance and t-tests between the treated groups was performed using a p-value of 0.05 and significance was determined using FDR correction (Benjamini and Hochberg,

1995). Proteins which had non-existent values (suggestive of absence or very low abundance in a sample) were also used in statistical analysis. Proteins found to be absent (below the level of detection) in one or more treatments and present (above the level of detection) in three or fewer treatments were also used in statistical analysis of the total differentially expressed group following imputation of the zero values with values that simulate low abundant proteins.

These proteins were subjected to Gene Ontology (GO) analysis by Blast2GO software tool (<https://www.blast2go.com/>). Statistically enriched GO descriptors were examined to identify clusters of proteins enriched within statistically significant differentially abundant (SSDA) protein lists ($P < 0.05$).

Statistical analysis

Experiments performed *in vitro* were carried out on three independent occasions, in four replicates and results are expressed as the mean \pm SE. Proteomic and yield data from *in vivo* testing had four replicates. Significant differences were considered at $P < 0.05$.

Results

Assessment of effect of *T. aggressivum* supernatants on *A. bisporus* growth *in vitro*

The effect of *T. aggressivum* supernatants on *A. bisporus* growth *in vitro* was assessed after 10-day incubation of *A. bisporus* at 25°C and additional 2-, 4- and 8-days of co-incubation with 25% v/v *T. aggressivum* filter-sterilized 48h supernatant or MEB (control). After two days, *T. aggressivum* supernatant inhibited growth by 27.9% where average growth for the control was

1.64±0.22 g and for the treatment was 1.18±0.11 g (Figure 1). After four days, *T. aggressivum* supernatant statistically significantly inhibited growth by 48% ($P<0.05$, $t=3.807$, $df=6$), where average growth for the control was 2.12±0.22 g and for the treatment was 1.11±0.15 g (Figure 1). After eight days *T. aggressivum* supernatant inhibited growth by 13.16%, average growth for the control was 4.10±0.34 g and for the treatment was 3.56±0.29 g (Figure 1). Only four-day coincubation was found to significantly ($P=0.05$) inhibit *A. bisporus* growth (Figure 1).

Proteomic analysis of *A. bisporus* response after exposure to *T. aggressivum* supernatant *in vitro*

Proteomic analysis was performed on one set of samples, where growth of *A. bisporus* has been significantly affected. Proteins were extracted from four replicate samples of *A. bisporus* exposed to *T. aggressivum* supernatant for 4 days and prepared for label free quantitative proteomics. In total 17654 peptides were identified, representing 1226 proteins. According to Perseus analysis a total of 73 *A. bisporus* proteins in the *T. aggressivum* +4 day treatment were determined to be differentially abundant (analysis of variance [ANOVA], $P < 0.05$), 36 increased and 37 decreased in relative abundance with a fold change value of >1.5 . A total of 280 *A. bisporus* proteins were deemed exclusive (i.e., with LFQ intensities present in all four replicates of treatment and absent in all four replicates of control). These proteins were also used in statistical analysis of the total levels corresponding to the differentially expressed group members following imputation of the zero values as described above. After data imputation, these proteins were included in the subsequent statistical analysis.

A principal-component analysis (PCA) performed on all filtered proteins distinguished the control and *T. aggressivum* -treated samples (Figure S4). Label free proteomic analysis of changes in the relative abundance of *A. bisporus* proteins, compared to the control, following exposure of cells to *T. aggressivum* supernatant was performed, and results indicated that exposure lead to an oxidative stress response (proteins that increased in relative abundance: zinc ion binding (+ 6.6 fold); peroxidase activity (+5.3 fold); carboxylic ester hydrolase (+2.4 fold); biosynthetic process (+3.1 fold); 60S ribosomal protein L36 (+3.3 fold); dipeptidase (+3.2 fold); [2Fe-2S] cluster assembly (+3.3 fold)) (Figure 2).

The proteins that were decreased in relative abundance in *A. bisporus* exposed to *T. aggressivum* supernatant were associated with growth: structural constituent of ribosome, translation (-12 fold), deadenylation-dependent decapping of nuclear-transcribed mRNA (-3.4 fold), superoxide dismutase (-3 fold), metal ion binding (-2.7 fold), small GTPase mediated signal transduction (-2.6 fold), deoxyribonucleotide catabolic process (-2.6 fold), GTP binding (-2.7 fold), glycine cleavage system P protein (-2.3 fold), and proteasome subunit beta (-2.3 fold) (Figure 2).

Proteins were subjected to Gene Ontology (GO) analysis by Blast2GO software tool. Several GO terms belonging to biological process (oxidation-reduction response to stress, metabolic and biosynthetic process), molecular function (ion binding, catalytic, hydrolase and oxidoreductase activity, structural constituent of ribosome and organic cyclic compound binding), cellular component (intracellular and non-membrane-bounded organelle, ribonucleoprotein complex and intrinsic component of membrane) were significantly enriched within the dataset. Hydrolases and oxidoreductases were the most enriched enzymes categories among proteins identified in *A. bisporus* treated with *T. aggressivum* (Figure S 5a, b, c, d).

Effect of *T. aggressivum* inoculum level on *A. bisporus* *in vivo*

The impact of *T. aggressivum* inoculum dilution level (10^{-3} and 10^{-4}) added to uninfected Bulk Phase III mushroom substrate on mushroom yield and disease symptoms of green mould on casing/compost, and fruiting bodies was assessed.

Average total yield (n=4) after 1st and 2nd flush was 4600 g_{xplot}⁻¹ in controls, 2900 g_{xplot}⁻¹ and 1100 g_{xplot}⁻¹ for 10^{-4} and 10^{-3} inoculation treatments, respectively (Figure 3). Inoculum dilution level influenced mushroom yield, with reductions in yield of 37 % at 10^{-4} level and 76 % at 10^{-3} level of infection.

Green mold was observed on inoculated plots starting from day 14, characterized by dense white mycelium that changed colour to green after extensive sporulation (Figure S6). Symptoms (spots and deformities) on fruiting bodies were noted on day 17 during the first flush (Figure S7). The 10^{-4} *T. aggressivum* inoculum decreased the yield by 29% to 56% after the first and second flush, respectively, and 10^{-3} *T. aggressivum* inoculum decreases the mushroom yield by 68% to 100% (Figure 3, 4). Colorimetry measurements (Chroma Meter CR-400, Konica Minolta, Sensing, INC., Japan) showed no statistical difference in colour quality among non-symptomatic mushrooms from the infected or control plots (Table S1). Colorimetric assay was mostly used in the Teagasc Mushroom Growing Unit in Dublin for measuring the difference in pilei color caused by Mushroom Virus X. Preliminary test showed that the green mould cannot be detected by colorimetric measurements. According to our results there is no significant difference in colour that can be used as a future detection method and overall green mould did not cause significant changes in colorimetric parameters.

Proteomic analysis of *A. bisporus* response after exposure to *T. aggressivum* in a mushroom growing room (*in vivo*)

A. bisporus proteins were extracted from Phase III mushroom substrate which had been infected with *T. aggressivum* at two different concentrations, 10^{-3} and 10^{-4} inoculum ($n = 4$). Phase III substrate samples were taken after 10-, 15-, 20-, and 25- days growth over the course of a mushroom crop cycle. No proteins matching either *A. bisporus* or *Trichoderma* database were found in samples up to day 20, possibly due to the complex nature of the substrate, which contains multiple microbiome proteins and high levels of organic matter that may have masked the *A. bisporus* proteins. It was possible to extract *A. bisporus* proteins from samples obtained on days 20 and 25. The results obtained on day 25 indicated that a total of 35 peptides were identified, representing 10 proteins. According to Perseus analysis a total of 7 *A. bisporus* proteins in *T. aggressivum* 10^{-4} infection level compost were determined to be differentially abundant (analysis of variance [ANOVA], $P < 0.05$), 1 increased and 6 decreased in relative abundance with a fold change value of >1.5 . One *A. bisporus* protein was deemed exclusive (i.e., with LFQ intensities present in all four replicates of treatment and absent in all four replicates of control) and was used in statistical analysis of the total levels corresponding to the differentially expressed group members following imputation of the zero values as described above. After data imputation, this protein was included in the subsequent statistical analysis, and results indicated that exposure lead to decrease in relative abundance of proteins involved in hydrolase activity (+ 4.3 fold) (Figure S8).

Label free proteomic analysis of changes in the abundance of *A. bisporus* proteins at day 25, following exposure of cells to *T. aggressivum* 10^{-3} inoculum was performed, and in total 41

peptides were identified, representing 10 proteins. According to Perseus analysis a total of 5 *A. bisporus* proteins in this *T. aggressivum* treatment were determined to be differentially abundant (analysis of variance [ANOVA], $P < 0.05$), 4 increased and 1 decreased in relative abundance with a fold change value of >1.5 . Results indicated that exposure lead to an oxidative stress response (proteins that increased in relative abundance: kinase activity (+2.6 fold), magnesium ion binding +2.6 fold), potassium ion binding, pyruvate kinase activity (+2.6 fold); carbohydrate metabolic process and beta-galactosidase activity (+2.2 fold); hydrolase activity [GO:0016787] (+2.1 fold); and hydrolase activity [GO:0016787] (+1.8 fold)) (Figure S9). The protein that decreased in relative abundance in *A. bisporus* treated with *T. aggressivum* 10^{-3} inoculum was involved in polysaccharide catabolic process, and the lyase activity (-3 fold) (Figure S9).

Proteins were subjected to Gene Ontology (GO) analysis by the Blast2GO software tool. Several GO terms belonging to molecular function (hydrolase activity), cellular components (intrinsic component of membrane) were significantly enriched within the datasets in both levels of infection. Hydrolases and oxidoreductases were the most enriched enzymes in 10^{-4} inoculated plots whereas in 10^{-3} infected plots hydrolases and transferases were most abundant amongst proteins identified in *A. bisporus* treated with *T. aggressivum* (Figure S10 a, b, c).

Discussion

The aim of the work presented here was to assess the response of *A. bisporus* to exposure to *T. aggressivum* both *in vitro* and *in vivo* in order to gain a better understanding of how they interact and why *A. bisporus* growth is so inhibited. The reduction in biomass caused by exposure of *A. bisporus* to *T. aggressivum* supernatant in the laboratory correlated with the yield

decrease in mushroom growing facility following the green mould infection. *In vitro* results showed that *T. aggressivum* supernatants reduced *A. bisporus* growth at all time points, but the statistically significant ($P = 0.05$) decrease of 48 % was found after four days of co-incubation. The cause of the reduced effect of mould supernatant may be due to the absence of a live organism in the supernatant and *A. bisporus* ability to overcome the impact of the one-time addition of supernatant. The transient effect of supernatant is mirrored in the proteomics analysis performed on 2- and 8-day (Fig S11 a,b) samples which showed minor changes vs control. However, proteomic response of mycelia after 4-day co-incubation with *T. aggressivum* supernatant (Figure 2) exhibited increases in proteins associated with oxidative stress response and distinguishes from the control. *In vivo* experiments, with continuous exposure to *T. aggressivum*, demonstrated that mushroom yield is vulnerable to both levels of *T. aggressivum* infection and the extent of crop loss directly correlates with the green mould infection level. A higher dose of *T. aggressivum* caused more severe disease symptoms in the substrate, on the crop surface and on fruiting bodies and resulted in lower yield, with reductions of 37 % at 10^{-4} level and 76 % at 10^{-3} level of infection compared to the controls.

Proteomic assessment revealed that both groups of samples, laboratory harvested wet mass (*in vitro* experiments) and mushroom growing compost samples (*in vivo* experiments), showed *A. bisporus* increased abundance of oxidative stress proteins (peroxidase, hydrolase, kinase and ion binding activity) when cultured in the presence of *T. aggressivum*. Also, proteins associated with growth were found to be reduced in abundance (polysaccharide catabolic process, structural constituent of ribosome, translation, deadenylation-dependent de-capping of nuclear-transcribed mRNA, small GTPase mediated signal transduction, deoxyribonucleotide catabolic process, GTP binding, glycine cleavage system P protein and proteasome subunit beta),

indicating that the two fungal organisms not only compete for space and nutrients (Williams *et al.* 2003) but do interact more as pathogen and host.

T. aggressivum does produce secondary metabolites, and volatile organic compounds found to negatively affect mushroom yield (Kredics *et al.* 2010). However, *A. bisporus* extracts have been found to promote *T. aggressivum* growth and conidia germination which was not seen in other species of genus *Trichoderma* (Mamoun *et al.* 2000; Mumpuni *et al.* 1998). Diverse opinions about the *T. aggressivum* f. *europaeum* development in compost could be found. Thus, *T. aggressivum* mycelium is barely visible in compost without the presence of *A. bisporus*, suggesting that aggressive *Trichoderma* needs the presence of *A. bisporus* to stimulate growth (Seaby *et al.* 1996; Rinker 1996; Romaine *et al.* 1996). Mamoun *et al.* (2000), discovered that the mycelium of *A. bisporus* is required for the induction of intensive sporulation of *T. aggressivum* f. *europaeum*.

The *Trichoderma* genus stands out among other Ascomycotina members for its abilities to synthesize numerous secondary metabolites, *in vivo* and *in vitro*, and many of its enzymes are used in industrial and biotechnology processes (Sivasithamparam and Ghisalbert 1998; Kubicek *et al.* 2009). In accordance with EU regulations for environmental protection and health, the Sustainable Use of Pesticides Directive (SUD) 2009/128/EC regulates the use of pesticides in agriculture and advocates an integrated pest management (IPM) approach to pesticide use whereby non-chemical biocontrol approaches to pest and disease control should be used where possible. Some *Trichoderma* spp. with antagonistic antifungal properties are used as biocontrol agents in plant protection since early 1930s (Weindling 1932; Harman *et al.* 2010; Lorito *et al.* 2010). Among the first such antifungal biocontrol substances isolated from certain *Trichoderma* spp. was 6-pentyl- α -pyrone, compound that has been synthetically made after it was first isolated

from *T. viride* (Collins and Hilim, 1972). The isolated volatile compound has a characteristic coconut smell and is present in *T. viride*, *T. atroviride* and *T. koningii* (Bisby, 1939; Dodd *et al.* 2003). While the antifungal properties of *Trichoderma* spp may be beneficially used for the control of fungal pathogens of plant-based crops, they are not desirable in mushroom crop production, where the crop is itself a fungus. Other mushroom crops such as *Pleurotus ostreatus* and *Lentinula edodes* also suffer from *Trichoderma* infections, highlighting the aggressive antifungal nature of this genus (Qiu *et al.* 2017; Wang *et al.* 2016).

Previous studies have suggested that *T. aggressivum* is a competitor for space and is a saprophytic contaminant in compost for mushroom cultivation rather than mycoparasite (Williams *et al.* 2003, O'Brien *et al.* 2014). Even though *T. aggressivum* produces stress tolerance and longevity proteins when in contact with *A. bisporus* and Phase 3 mushroom substrate (O'Brien *et al.* 2014; Williams *et al.* 2003; O'Brien *et al.* 2017; Hatvani *et al.* 2010) it should be considered as a true mycopathogen as the results presented here show a direct effect of *T. aggressivum* supernatant and hyphae on *A. bisporus* development and the induction of an oxidative stress response. This understanding is an important epidemiological insight further unmasking the *T. aggressivum* diseases processes which may enable development of novel ways of prevention, control and treatment of this serious disease of the mushroom crop.

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Figure legends

Figure 1. *A. bisporus* growth [g] in CYM after 12-, 14- and 18-days incubation at 25 °C, containing 25% v/v of either: MEB (Control) or *T. aggressivum* 48h supernatant (+ *T. aggressivum* SN) for the last 2-, 4- and 8-days of incubation (+2, +4 +8). 12-days: $P=0.116$, $t=1.836$, $df=6$; 14-days: ** $P=0.01$, $t=3.807$, $df=6$; 18-days: $P=0.258$, $t=1.217$, $df=8$.

Figure 2. Proteomic responses of *A. bisporus* following 4 days incubation with 25% v/v 48h supernatant of *T. aggressivum*. Volcano plot represent protein intensity difference ($-\log_2$ mean intensity difference) and significance in differences ($-\log P$ -value) based on a two-sided t-test. Proteins above the line are considered statistically significant (P value < 0.05) and those to the right and left of the vertical lines indicate relative fold changes > 1.5 . Annotations are given for the most differentially abundant proteins identified. These plots are based upon post imputed data.

Figure 3. Average yield [$\text{g}_{\text{xplot}}^{-1}$] after first, and second flush and overall average yield depending on the infection level (10^{-3} and 10^{-4}) compared to the uninfected control plots ($p = 0.2456$).

Figure 4. Mushroom productivity during the first flush. A - Control; B - 10^{-4} inoculation rate; C - 10^{-3} inoculation rate. Note the presence of green mould, diseased mushrooms and reduced cropping area in 10^{-4} and 10^{-3} inoculation treatments compared to the control.

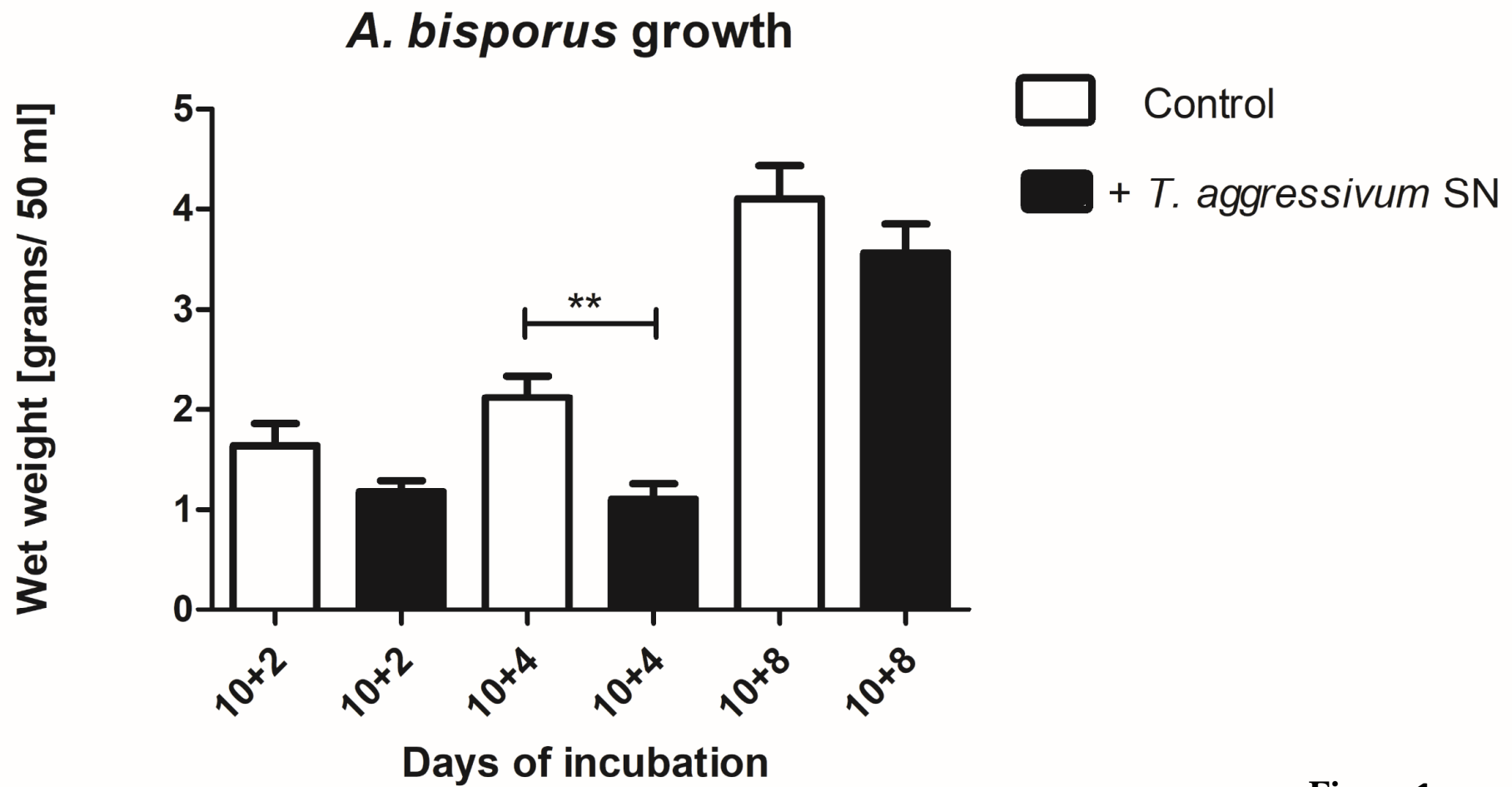


Figure 1.

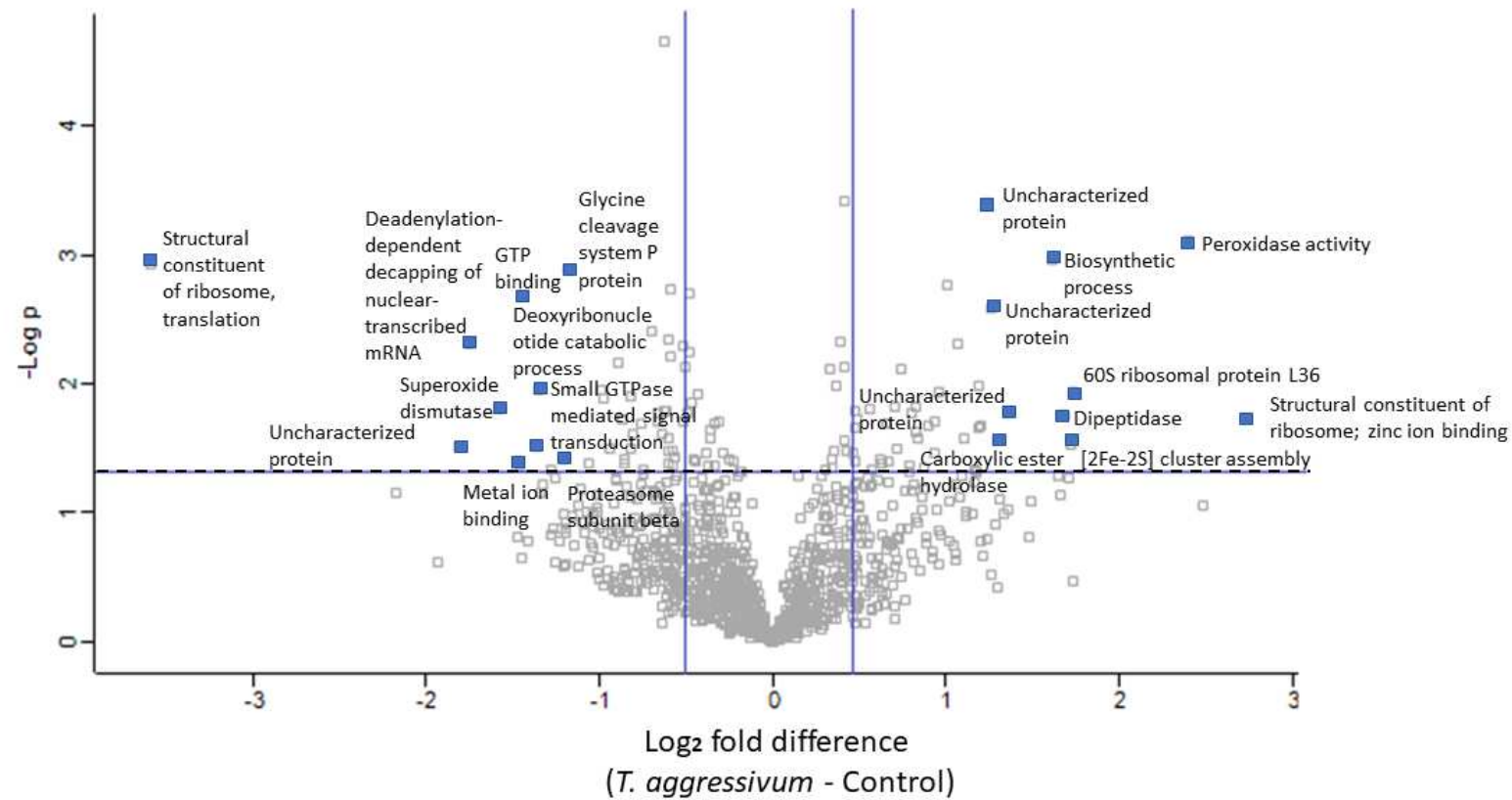
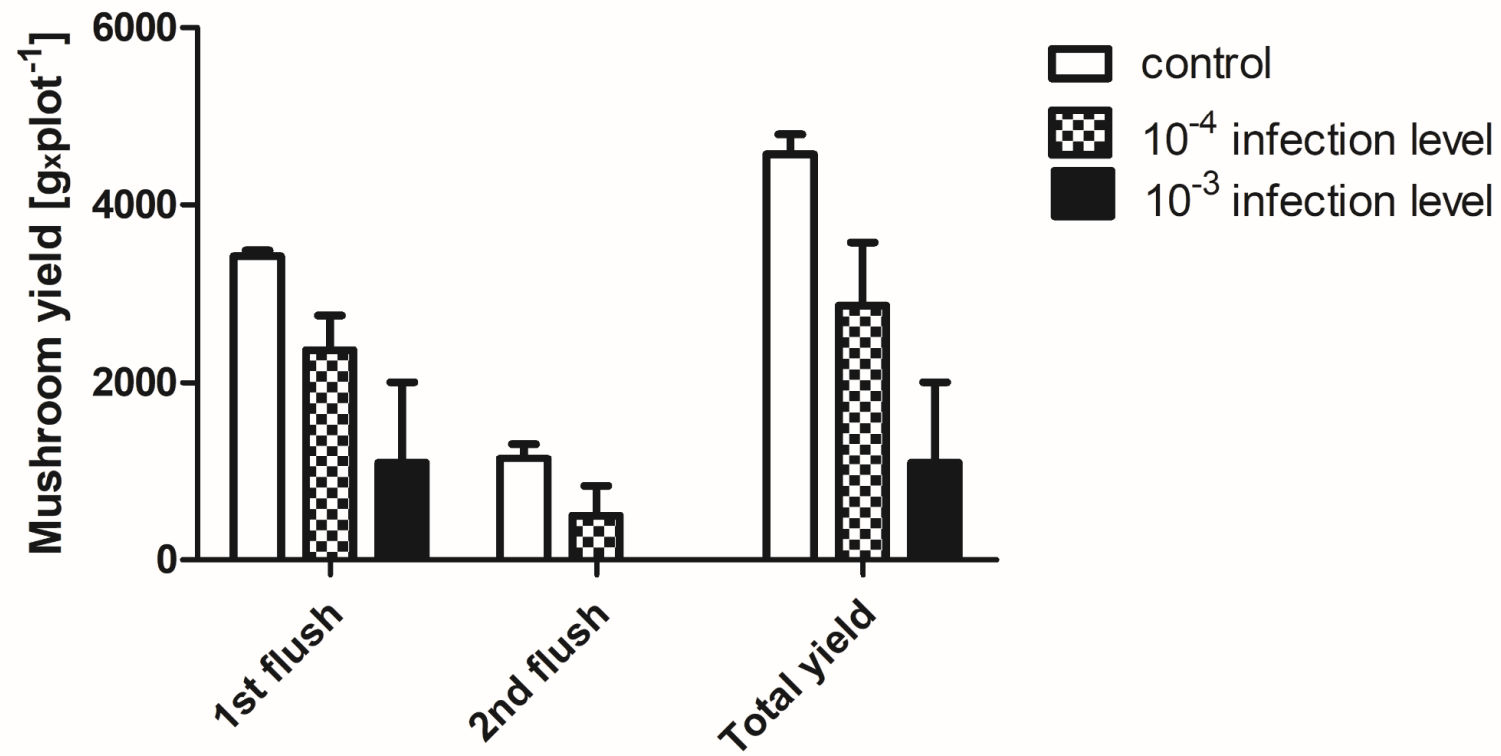


Figure 2.

**Figure 3.**

**Figure 4.**

Highlights

1. Exposure of *A. bisporus* to *T. aggressivum* supernatant induces oxidative stress.
2. Exposure to *T. aggressivum* *in vivo*, leads to oxidative stress in *A. bisporus*.
3. Co-culture of *A. bisporus* and *T. aggressivum* reduces mushroom yield.
4. *A. bisporus* and *T. aggressivum* interact as host-pathogen, not competitors.